Both linoleic and α-linolenic acid prevent insulin resistance but have divergent impacts on skeletal muscle mitochondrial bioenergetics in obese Zucker rats

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Mitochondrial dysfunction is a central hypothesis in the progression of skeletal muscle IR and is characterized traditionally by reduced content or impairment of function affecting rates of FAO (26). However, given that increased mitochondrial content can parallel the development of IR (15, 19, 53) and that the capacity of ATP production far exceeds reductions in content (16, 21), the relationship between mitochondrial dysfunction and IR remains largely unresolved. Recently, alterations in mitochondrial bioenergetics have also been associated with increased reactive oxygen species (ROS) emission-induced IR (2), whereas pharmacological and genetic approaches that increase antioxidants prevent diet-induced IR (6, 35). Therefore, therapies that improve mitochondrial oxidative phosphorylation or reduce mitochondrial ROS emission may be particularly advantageous. Although n-3 PUFA appear ideal in mitigating IR, improved insulin sensitivity with EPA and DHA occurs independent of improvements in mitochondrial content or function (33) and is known to increase mitochondrial susceptibility to oxidative damage (i.e., lipid peroxidation) and propensity to emit ROS (33). Clearly, the mechanistic relationship between long-chain n-3 PUFA and insulin sensitivity remains to be fully delineated.

Compared with EPA and DHA, little is known about the relationship between IR and α-linolenic acid (ALA). Although ALA can be endogenously converted into EPA/DHA, tracer studies have revealed that the conversion efficiency is low (<8%) (44, 54). Therefore, it is conceivable that ALA and EPA/DHA have divergent effects on insulin sensitivity, although this remains to be shown. In contrast, n-6 PUFA have traditionally been viewed as detrimental to insulin sensitivity in part because they serve as precursors for the production of proinflammatory eicosanoids (14); however, this view has been challenged, as accumulating evidence suggests that not all n-6 PUFA are proinflammatory (25). Interestingly, linoleic acid (LA) may also influence mitochondrial function, as it is the predominant fatty acyl moiety in the mitochondrial-specific phospholipid species cardiolipin (28). Current estimates suggest that n-6 PUFA are consumed in five- to 20-fold greater amounts than n-3 PUFA (7); however, the health benefits of LA supplementation remain ambiguous. This highlights the need to study LA and the mechanisms by which it may influence IR in obesity.

Therefore, we investigated in young obese Zucker rats whether LA- and ALA-enriched diets could prevent the expected age-related decline in glucose homeostasis. Skeletal muscle mitochondria exist in two spatially distinct subpopulations known as subsarcolemmal (SS) and the predominant intermyofibrillar (IMF) mitochondria. These subpopulations possess unique characteristics (5, 17, 38, 41) and respond
differently to various metabolic perturbations in obesity and type 2 diabetes (9, 19, 45) as well as changes in diet composition (8, 36). Therefore, we also determined subpopulation-specific responses of SS and IMF mitochondria to LA- and ALA-enriched diets and the necessity of adaptations within these mitochondria in mitigating IR. Altogether, our data suggest that both LA and ALA prevented impairments in whole body glucose homeostasis consistently seen with obese Zucker rats and have differential effects on SS mitochondrial protein content and function.

MATERIALS AND METHODS

Animals. Five-week-old male lean (n = 48) and obese (n = 48) Zucker rats were purchased from Charles River Laboratories. Animals were housed in a temperature-regulated room on a 12:12-h light-dark cycle with water available ad libitum. Control animals were given unrestricted access to a control diet, whereas treated animals within each genotype were pair-fed to match for caloric content. After 12 wk, animals were randomly assigned either to determine whole body and muscle-specific insulin sensitivity (n = 6) or for assessments of mitochondrial bioenergetics (n = 10). Anesthesia (60 mg/kg pentobarbital sodium injection), animal care, and housing procedures were approved by the University of Guelph Animal Care Committee.

Diets and feeding. All diets used in the present study were purchased through Research Diets (New Brunswick, NJ). Daily food consumption of lean and obese rats fed the control diet (no. AIN-93G; 20% protein, 64% carbohydrate, and 16% fat) was recorded by weight after pair-fed rats given LA- (no. AIN-93G + 10% safflower oil; 20% protein, 54% carbohydrate, and 26% fat) and ALA-supplemented (no. AIN-93G + 10% flaxseed oil; 20% protein, 54% carbohydrate, and 26% fat) diets. Diet fatty acid composition was confirmed by gas chromatography.

Whole body glucose and insulin tolerance. Four-hour-fasted animals underwent an intraperitoneal glucose (IPGTT; 2 g/kg) and insulin tolerance test (IPITT; 1.0 U/kg) separated by 48 h, as described previously (23).

Muscle-specific insulin signaling. To determine the phosphorylation of proteins involved in insulin-mediated signaling by Western blotting (described below), muscle was excised before and 15 min after an intraperitoneal insulin injection (1.0 U/kg) and rapidly frozen in liquid nitrogen.

Skeletal muscle mitochondrial isolation. Isolation of SS and IMF mitochondria was achieved by differential centrifugation. The respective speeds of centrifugation at each step were adapted from previous work (11) as well as the chemical composition of the isolation buffer (52). The exact protocols used in the present study were reported previously (32).

Mitochondrial bioenergetics. Rates of mitochondrial oxygen consumption and mitochondrial hydrogen peroxide (H2O2) emission were measured, as reported previously (32). In addition, separate experiments were performed to measure rates of oxygen consumption in the presence of 25 μM palmitoyl-CoA + 2 mM malate + 750 μM l-carnitine. A submaximal (100 μM) ADP concentration was used to determine per unit oxygen (P/O) ratios and a saturating ADP concentration (5 mM) to determine maximal palmitoyl-CoA-driven respiration.

Western blotting. Whole muscle homogenate (n = 6) were isolated from lean and IMF mitochondrial samples were separated by electrophoresis using SDS-PAGE, transferred to polyvinylidene difluoride membranes, and quantified, as reported previously (32). The following commercially available antibodies were used: total and phosphorylated (Thr308 and Ser473) Akt (Cell Signaling Technology), total and phosphorylated (Thr642) AS160 (Cell Signaling Technology), MitoProfile Total oxidative phosphorylation (OXPHOS) antibody cocktail (MitoSciences), adenosine nucleotide translocase 1 (ANT1; MitoSciences), ANT2 (Abcam, Cambridge, MA), manganese superoxide dismutase 2 (SOD2; Abcam), uncoupling protein 3 (UCP3; Abcam), and 4-hydroxynonenal (4-HNE; Alpha Diagnostics). All samples were detected from the same Western blot by cutting gels and transferring onto a single membrane to limit variability. Equal loading of protein was verified using Ponceau staining.

Protein carbonylation. The commercially available Oxyblot Protein Oxidation Detection Kit (Millipore; Billerica, MA) was used to assess protein carbonylation, as described previously (40).

Statistics. A one-way ANOVA, followed by a Newman-Keuls multiple comparison post hoc analysis, was used to determine the effects of LA and ALA supplementation within genotypes. It was determined that diets did not affect markers of interest in lean animals, thus permitting the use of an unpaired Student’s t-test to compare diet-matched lean and obese Zucker rats for subsequent analyses (Figs. 1–7). P ≤ 0.05 was considered statistically significant.

RESULTS

LA and ALA maintain whole body glucose homeostasis. LA and ALA did not alter glucose or insulin tolerance in lean animals (Fig. 1, A and C). In contrast, obese control rats had elevated fasting blood glucose compared with control lean rats (13.9 ± 2.1 vs. 5.0 ± 0.2 mM), which resulted in an increased AUC during both glucose and insulin intolerance tests. However, when the baseline values were adjusted to take into consideration the obesity-related increase in fasting blood glucose, consumption of both ALA and LA prevented glucose and insulin intolerance in obese animals (Fig. 1, C–F). Specifically, the baseline value during the IPGTT was constrained as the lowest individual glucose concentration within each genotype (lean = 3.9 mM; obese = 5.5 mM) (Fig. 1E), whereas during the IPITT, individual baseline values were set as the lowest blood glucose value of each animal. This method adjusts for the elevated basal glycemia of obese control rats, allowing for a more concrete assessment of glucose and insulin action independent of fasting blood glucose levels. Accordingly, we report that obese control rats exhibit a substantially greater AUC during both glucose (+70%) and insulin challenges (+84%) relative to lean controls, whereas no differences were observed between diet-matched animals fed LA and ALA (Fig. 1, E and F). Altogether, these data suggest that both LA and ALA prevented the development of insulin resistance in obese Zucker rats.

LA and ALA preserve skeletal muscle insulin signaling. Several parameters can influence whole body glucose and insulin tolerance independent of skeletal muscle insulin sensitivity (e.g., glucose/insulin actions within adipose tissue, liver, and pancreas). Therefore, it was important to determine specifically skeletal muscle insulin sensitivity in obese animals following LA and ALA supplementation. To determine this, we next investigated the ability of insulin to induce phosphorylation of proteins involved in the canonical insulin-signaling cascade. Within lean and obese animals there were no differences in total content of Akt and AS160 protein (Fig. 2A). In obese control animals, insulin failed to stimulate phosphorylation of Akt at Ser473 (Fig. 2B) and Thr308 (Fig. 2C), as well as AS160 at Thr642 (Fig. 2D), above basal levels (Fig. 2A). In contrast, obese rats supplemented with ALA maintained insulin-induced phosphorylation of Akt Ser473 (+100%) and Thr308 (+75%) as well as AS160 Thr642 (+40%) (Fig. 2, B–D). Although LA evoked similar improvements in Akt

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phosphorylation at both sites, no changes were seen with AS160 (Thr642). These data, in combination with the IPITT results, suggest that both LA and ALA maintain skeletal muscle insulin signaling in obese Zucker rats.

ALA preferentially increases electron transport chain proteins in SS mitochondria. The accumulation of OXPHOS proteins in whole muscle extracts was not different in obese rats following ALA or LA supplementation compared with controls (Fig. 3, A–F). Therefore, we reexamined OXPHOS protein content in purified SS and IMF mitochondrial fractions. Compared with lean controls, obese control rats showed a significant increase (+100%) in ATP synthase content in SS mitochondria only (Fig. 4, A and F). Interestingly, SS mitochondria from obese ALA rats showed significant increases in complex I subunit NUDFB8 (+100%), complex III subunit core 2 (+80%), and ATP synthase (+150%) (Fig. 4, A, B, D, and F). In contrast, the content of electron transport chain (ETC) markers in mitochondria isolated from LA-supplemented rats appeared identical to diet-matched leans (Fig. 4, A–F), suggesting an absence of compensatory adaptations. Finally, IMF mitochondria remained constant for all OXPHOS protein targets measured across all groups (Fig. 4, A–F), likely

Fig. 1. Intraperitoneal glucose (IPGTT) and insulin tolerance tests (IPITT) for lean (A and C, respectively) and obese rats (B and D, respectively). ●, Control (Cont) diet; ▽, α-linolenic acid (ALA) diet; △, linoleic acid (LA) diet. Area under the curve (AUC) values for IPGTT (E) and IPITT (F). Data expressed as means ± SE; n = 6 for each measure. +Significantly different from obese control (P < 0.05); *significantly different from diet-matched lean animals (P < 0.05).
accounting for the inability to detect SS mitochondrial adaptations at the whole muscle level.

LA prevents compensatory bioenergetic adaptations in obesity. We next determined whether mitochondrial bioenergetics were altered by measuring rates of oxygen consumption and H$_2$O$_2$ emission in isolated SS and IMF mitochondria. To confirm the integrity of our isolation protocol, mitochondrial ADP consumed P/O and respiratory control ratios, as well as absolute values of state 3 and 4 respiration, are presented in Table 1. Following analysis it was determined that mean values for all lean animals did not vary significantly; therefore, for simplicity, we present subsequent analyses as a percent change corresponding to diet-matched lean animals.

We measured mitochondrial pyruvate- and palmitoyl-CoA-supported respiration as a primary assessment of respiratory function. Compared with lean animals, obese controls exhibited significant increases in maximal ETC capacity (complex I and complex I + II) of SS mitochondria only (Fig. 5, B–F), which was analogous to that observed in ATP synthase protein content (Fig. 4). Similar adaptations were seen in obese ALA rats compared with their lean counterparts, including a significant increase in pyruvate-supported state 4 respiration (Fig. 5A). In contrast, respiration of SS mitochondria from obese LA rats was identical to lean animals, thus fitting with the observed expression of OXPHOS proteins. Rates of oxygen consumption in IMF mitochondria were similar in all groups and diets. Altogether, it appears that the improvements in whole body and muscle-specific insulin sensitivity conferred by ALA and LA are associated with distinct impacts on mitochondrial content and function.

Mitochondrial H$_2$O$_2$ emission and markers of oxidative stress. We next determined whether PUFA supplementation was associated with a reduced mitochondrial H$_2$O$_2$ emission and oxidative stress. Obese control rats did not display increased SS or IMF mitochondrial H$_2$O$_2$ emission (Fig. 6A),

Fig. 2. Skeletal muscle insulin-signaling proteins in basal state and following insulin injection. A: representative blots of total and phosphorylated (p)-Akt. B: Ser$^{473}$. C: Thr$^{308}$. D: Akt substrate of 160 kDa (AS160) Thr$^{642}$. Data for insulin-stimulated phosphorylation are expressed as means ± SE of the %change from the basal state; n = 6 for each measure. *Significantly different from basal state of the same animal (P < 0.05).
whereas ALA increased maximal H₂O₂ emission rates in SS mitochondria by 80% (Fig. 6A). When expressed relative to absolute state 4 respiration values (Table 1), diet-specific differences in H₂O₂ emission were abolished and resembled that of lean animals. Interestingly, a recent study showed that EPA/DHA supplementation in mice on a high-fat diet increased ROS emission in isolated mitochondria using a similar approach (33). Therefore, we sought to rule out the contribution of EPA/DHA-derived lipid radicals, which could potentially interact with amplex red to artificially increase background fluorescence. Using purified EPA and DHA at concentrations known to exist in rat mitochondria, we found that increased H₂O₂ emission following ALA supplementation was not a methodological artifact (data not shown). In addition, LA supplementation did not alter maximal H₂O₂ emission in obese animals (Fig. 6A). To assess the implications of the change in maximal H₂O₂ emission, we quantified protein carbonyls and 4-HNE content (a marker of lipid peroxidation) in whole muscle extracts and in isolated mitochondria. Protein oxidation was not altered significantly by genotype or by diet in whole muscle extracts (Fig. 6B) or in isolated mitochondrial fractions (Fig. 6C). In contrast, these analyses revealed that, despite unaltered rates of mitochondrial H₂O₂ emission, obese control animals have a significant increase in total muscle lipid peroxidation (+30%), suggesting the presence of oxidative stress (Fig. 6D). Furthermore, 4-HNE was increased ~70% within

Fig. 3. Skeletal muscle homogenate mitochondrial oxidative phosphorylation (OXPHOS) proteins. A: representative blots reveal no changes in complex 1 subunit NDUFB8 (B), complex II subunit 30 kDa (C), complex III subunit core 2 (D), complex IV subunit 1 (E), or ATP synthase α-subunit (F) of obese animals compared with diet-matched lean animals; n = 6 for each measurement. Data are expressed as means ± SE.
LA mitochondria of obese controls but was reduced in SS mitochondria (Fig. 6E). Overall, in obesity, LA prevented changes in 4-HNE content within whole muscle (Fig. 6D) and SS/IMF mitochondria (Fig. 6E), resembling lean healthy animals. ALA supplementation also prevented increases in 4-HNE content within whole muscle (Fig. 6D) and IMF mitochondria (Fig. 6E) seen in obese control rats.

Mitochondrial ADP transport, uncoupling, and antioxidant proteins. Given the apparent discrepancy between maximal in vitro mitochondrial H$_2$O$_2$ emission rates and in vivo markers of oxidative stress, we next examined the expression of proteins known to influence mitochondrial H$_2$O$_2$ emission. ANT1 content did not change in SS mitochondria across all groups and diets (Fig. 7A). In contrast, ANT1 content in IMF mitochondria of obese controls but was reduced in SS mitochondria (Fig. 6E). Overall, in obesity, LA prevented changes in 4-HNE content within whole muscle (Fig. 6D) and SS/IMF mitochondria (Fig. 6E), resembling lean healthy animals. ALA supplementation also prevented increases in 4-HNE content within whole muscle (Fig. 6D) and IMF mitochondria (Fig. 6E) seen in obese control rats.

Fig. 4. Changes in OXPHOS proteins of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria. A: representative blots for complex 1 subunit NDUFB8 (B), complex II subunit 30 kDa (C), complex III subunit core 2 (D), complex IV subunit 1 (E), and ATP synthase α-subunit (F); n = 10 for each measurement. Data are expressed as means ± SE. *Significantly different from diet-matched lean animals (P < 0.05).
content in SS mitochondria of obese controls showed a trend-

P content in SS mitochondria of obese controls showed a trend-

FIG. 7B

of LA and ALA supplementation decrease 50% in SS mitochondria of obese controls; however, this change was prevented with LA and ALA supplementation decrease 50% in SS mitochondria of obese controls; however, this change was prevented with LA and ALA supplementation. Overall, the current data support a beneficial link between LA and insulin sensitivity and provide novel evidence that LA can prevent impairments in glucose homeostasis and skeletal muscle insulin sensitivity in a model of severe genetic obesity.

**ALA attenuates the development of whole body glucose homeostasis.** Obese Zucker rats display normal glucose and insulin tolerance at 5 wk of age (56); however, when the current dietary intervention was initiated, they rapidly displayed genetic obesity, hyperinsulinemia, hyperlipidemia, and peripheral insulin resistance. Therefore, obese Zucker rats represent an attractive model to investigate nutritional approaches that prevent the development of IR (27). Although the benefits of fish oil on insulin sensitivity are supported extensively in the literature (31, 33, 39, 46, 50), we provide evidence that ALA, the precursor of EPA and DHA, may also be efficacious in improving insulin sensitivity. Recent work suggests that fish oil supplementation promotes the expression of OXPHOS proteins in ameliorating IR (33). The current data support this interpretation, as we observed similar increases in H2O2 emission, OXPHOS proteins, and catalase with ALA. In the absence of oxygen consumption in the presence (state 3) and absence (state 4) of ADP for isolated SS and IMF mitochondria. Diets groups are control, LA, or ALA. State 3 and 4 values are expressed as nmol-min⁻¹-mg⁻¹ mitochondrial protein. RCR (state 3/state 4) and ADP consumed per unit oxygen (P/O ratio) reflect mitochondrial integrity and coupling. *Significantly different from obese ALA (P < 0.05).

**DISCUSSION**

The current study shows that the development of IR in obesity can be prevented by dietary supplementation with LA and ALA. Strikingly, a moderate 10% isocaloric increase in either of these PUFA species was efficient in attenuating the impaired glucose homeostasis documented in a common genetic model of obesity and insulin resistance. These findings were associated with the conservation of skeletal muscle insulin signaling and oxidative stress relative to lean healthy animals. Examining aspects of mitochondrial dysfunction revealed that LA and ALA have markedly different impacts on SS mitochondrial ETC content and bioenergetics compared with IMF. This was further supported by ALA-specific increases in maximal H2O2 emission in SS mitochondria as well as the expression of catalase. Overall, the current data support from obese control rats increased significantly (+100%) but was comparable with a lean phenotype in obese LA and ALA groups (Fig. 7A). Relative to lean healthy animals, ANT2 was decreased 50% in SS mitochondria of obese controls; however, this change was prevented with LA and ALA supplementation (Fig. 7B). In obese rats, the abundance of UCP3 (Fig. 7C) in SS mitochondria was increased (P < 0.05) across all diet groups (control, +250%; ALA, +300%; LA, +500%). Finally, SOD2 content in SS mitochondria of obese controls showed a trending increase (P = 0.07) and was significantly elevated in IMF mitochondria (+50%). These changes were prevented by LA and ALA in obese rats (Fig. 7D), as SOD2 content was similar to lean animals. In contrast, catalase content in muscle homogenate was not altered in either obese control or LA groups but was increased significantly (+60%) in obese rats fed ALA (Fig. 7E).

**Table 1. Mitochondrial respiratory characteristics**

<table>
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<tr>
<th></th>
<th>Lean Control</th>
<th>Lean ALA</th>
<th>Lean LA</th>
<th>Obese Control</th>
<th>Obese ALA</th>
<th>Obese LA</th>
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<td></td>
<td></td>
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<tr>
<td>P/O ratio</td>
<td>2.47 ± 0.07</td>
<td>2.56 ± 0.08</td>
<td>2.53 ± 0.06</td>
<td>2.57 ± 0.08</td>
<td>2.49 ± 0.09</td>
<td>2.56 ± 0.05</td>
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<td>RCR</td>
<td>10.9 ± 1.21</td>
<td>9.6 ± 0.92</td>
<td>10.5 ± 1.21</td>
<td>13.2 ± 1.27</td>
<td>10.4 ± 1.11</td>
<td>12.9 ± 1.35</td>
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<td>State 4</td>
<td>24.5 ± 3.28</td>
<td>21.1 ± 1.68</td>
<td>25.1 ± 3.38</td>
<td>26.6 ± 3.79</td>
<td>30.5 ± 3.21</td>
<td>18.9 ± 1.60*</td>
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<td>184 ± 13.9</td>
<td>262 ± 39.5</td>
<td>327 ± 41.6</td>
<td>297 ± 42.7</td>
<td>234 ± 23.0</td>
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<td></td>
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<tr>
<td>P/O ratio</td>
<td>2.48 ± 0.08</td>
<td>2.49 ± 0.07</td>
<td>2.35 ± 0.07</td>
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<td>RCR</td>
<td>20.5 ± 3.44</td>
<td>16.2 ± 2.62</td>
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<tr>
<td>P/O ratio</td>
<td>2.27 ± 0.11</td>
<td>2.33 ± 0.10</td>
<td>2.26 ± 0.17</td>
<td>2.49 ± 0.12</td>
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<td>RCR</td>
<td>9.11 ± 1.02</td>
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<tr>
<td>P/O ratio</td>
<td>2.18 ± 0.22</td>
<td>2.26 ± 0.13</td>
<td>2.16 ± 0.14</td>
<td>2.65 ± 0.13</td>
<td>2.32 ± 0.13</td>
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<td>20.0 ± 16.1</td>
<td>21.7 ± 2.42</td>
<td>20.1 ± 3.94</td>
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<tr>
<td>State 3</td>
<td>187 ± 28.5</td>
<td>158 ± 28.9</td>
<td>173 ± 21.4</td>
<td>220 ± 42.4</td>
<td>229 ± 33.7</td>
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Data are presented as means ± SE; n = 10 for each measurement. P/O, per unit oxygen; RCR, respiratory control ratio; ALA, α-linolenic acid; LA, linoleic acid; SS, subsarcolemmal; IMF, intermyofibrillar. Absolute rates of oxygen consumption in the presence (state 3) and absence (state 4) of ADP for isolated SS and IMF mitochondria. Diets groups are control, ALA, or LA. State 3 and 4 values are expressed as nmol-min⁻¹-mg⁻¹ mitochondrial protein. RCR (state 3/state 4) and ADP consumed per unit oxygen (P/O ratio) reflect mitochondrial integrity and coupling. *Significantly different from obese ALA (P < 0.05).
prevented the development of whole body glucose intolerance and maintained muscle-specific insulin sensitivity and 4-HNE content in obese animals. In contrast to ALA, these improvements were independent of changes in H₂O₂ emission, OXPHOS proteins, and antioxidant enzyme expression, raising the possibility of a divergent mechanism for improving insulin sensitivity. However, our findings do not exclude the possibility that LA remodels the membrane cardiolipin profile within mitochondria (28), which is known to impact mitochondrial function (18, 28). Although previous reports have linked obesity with changes that would likely promote mitochondrial H₂O₂ emission, including increased ETC sensitivity to reducing equivalents (34) and diminished sensitivity to ADP (49), we found no changes in maximal ADP-stimulated respiration following LA supplementation. Therefore, it remains possible that LA may alter the dynamic response of mitochondria to

Fig. 5. Isolated SS and IMF mitochondrial respiration. Basal (A) and ADP-stimulated (B) pyruvate-supported respiration, maximal complex I (plus glutamate; C) and maximal electron transport chain respiration (plus succinate; D), and basal (E) and ADP-stimulated (F) states of palmitoyl-CoA respiration; n = 10 for each measurement. Data expressed as means ± SE. *Significantly different from diet-matched lean animals (P < 0.05).
Fig. 6. Markers of oxidative stress. A: maximal succinate H$_2$O$_2$ emission in SS and IMF mitochondria. B and C: protein carbonylation in muscle homogenate (B) and SS and IMF mitochondria (C). D and E: 4-hydroxynonenal (4-HNE) content in muscle homogenate (D) and SS and IMF mitochondria (E) indicates lipid peroxidation. Representative blots shown in respective panels; $n = 10$ for each measurement. Data are expressed as means ± SE. *Significantly different from diet-matched lean animals ($P < 0.05$).
Fig. 7. Changes in adenine nucleotide translocase 1 (ANT1; A), ANT2 (B), mitochondrial uncoupling protein 3 (UCP3; C), and manganese superoxide dismutase (SOD2; D) in SS and IMF mitochondria. For data shown in A–D, n = 10 for each measure. E: catalase content in muscle homogenate; n = 6 for all groups. Data are expressed as means ± SE. *Significantly different from diet-matched lean animals (P < 0.05).
submaximal substrate concentrations. Regardless of the elusive mechanism of action, the current data provide convincing evidence that LA prevented IR in obese Zucker rats.

Mitochondrial H$_2$O$_2$ emission and uncoupling and antioxidant proteins. The current study found increased ETC content only within SS mitochondria, which represents ~20% of total cellular mitochondrial volume (12, 20), accounting for the absence of changes in whole muscle measurements. The current study cannot explain mechanistically why SS mitochondria preferentially respond, although this appears to be a conserved observation across cellular stresses (19, 36). However, the increased expression of ETC proteins within the SS mitochondria likely contributed to the increase in maximal mitochondrial H$_2$O$_2$ emission, as normalization of emission rates to state 4 respiration negated all differences. Therefore, the increase in ETC subunits following ALA supplementation may be construed as a negative adaptation, as mitochondrial lipid uptake and ROS emission have been causally linked to IR (2), possibly through ROS-mediated activation of the NF-κB/IκB/IKKβ pathway, attenuating insulin signaling by serine phosphorylation of insulin receptor substrate 1 (48, 55). However, this working model remains controversial, as chronic mitochondrial antioxidant treatment that improves cellular redox balance does not improve insulin sensitivity following a high-fat diet (40). Perhaps subtle increases in mitochondrial ROS emissions are required for the transcriptional adaptations that are necessary to regulate metabolic homeostasis during a high-fat challenge. Our data indirectly support this model, as ALA supplementation increased maximal H$_2$O$_2$ emission and OXPHOS proteins in SS mitochondria as well as muscle catalase content. Our results are consistent with the suggestion that incorporating n-3 PUFA into mitochondrial membranes increases the propensity for ROS production (1, 33). Furthermore, in vivo markers of oxidative stress (4-HNE and protein carbonylation) suggest that despite an increase in maximal H$_2$O$_2$ emission, ALA supplementation conserved redox balance in whole muscle and mitochondrial samples. Given that ROS have several intracellular functions, including participation in the complex signaling network involved in mitochondrial biogenesis (22, 37), perhaps the increased OXPHOS expression within SS mitochondria in the obese ALA group is mediated in part through tightly regulated redox signaling.

In contrast, we found no differences in maximal H$_2$O$_2$ emission or catalase content in lean and obese animals fed LA. Unlike the changes seen with obese control and ALA groups, mitochondria from the obese LA group were identical to their lean counterparts, suggesting that compensatory bioenergetic adaptations are not necessary to preserve glucose homeostasis and muscle insulin signaling while consuming LA. Previous work showed that arachidonic acid increased mitochondrial ROS emission and was linked to mitochondrial dysfunction (10). Surprisingly, the precursor LA did not alter rates of mitochondrial H$_2$O$_2$ emission, and therefore, prevention of IR occurs through a mechanism not investigated in the current study. Although speculative, LA was shown to drive a lipoxigenase-mediated eicosanoid response, leading to production of the PPARα-activating 13-hydroperoxy-9,11-octadecadienoic acid (29). Therefore, if LA evokes a PPAR-mediated improvement in glucose homeostasis, the mechanism by which it acts may differ from that of n-3 PUFA. Alternatively, LA consumption may involve primary adaptations within the liver, pancreas, and white adipose tissue and secondary responses within muscle, a possibility that has not been explored in the current study. Regardless, the current study provides evidence that LA is beneficial at preventing the development of insulin resistance.

Perspectives and limitations. The current study provides insight on the link between ALA and insulin sensitivity and evidence that LA supplementation represents additional therapeutic potential. Although aspects of mitochondrial dysfunction were very similar between obese control and ALA-supplemented rats, the preservation of skeletal muscle insulin signaling and whole body glucose homeostasis highlights the value of this n-3 PUFA. The precise mechanism(s) by which LA and ALA exert their preserving effects requires further elucidation and may involve changes in liver given the enhanced glucose tolerance. Indeed, EPA/DHA may augment hepatic IR and lipotoxicity by increasing FAO, inhibiting de novo lipogenesis and reducing proinflammatory cytokine production (42). Furthermore, within white adipose tissue, these n-3 PUFAs are known to improve factors influencing IR, such as adipocyte morphology, rates of endogenous FAO, and adipokine secretory profiles, as well as immunometabolic status (as recently reviewed in Ref. 42). Whether LA and ALA exert their effects through similar mechanisms remains to be shown.

In the current study, we were unable to uncouple the effects of ALA from EPA and DHA; therefore, it is possible that the effects seen with ALA are due to its conversion (albeit limited) into EPA/DHA. Future work using animal models that prevent the conversion of ALA into EPA/DHA will enable us to more definitively describe the independent role of ALA on skeletal muscle insulin signaling. Also, in the current study, the macronutrient composition of the LA and ALA diets was out of necessity different from control diets, having higher fat (26 vs. 16% in control diet) and by default decreased carbohydrate content (54 vs. 64% in control diet). Therefore, future studies should also determine whether the modest 10% increase/decrease in dietary fat/carbohydrate could override the strong genetic predisposition for an IR phenotype.

More importantly, our data challenge the traditional view that LA is harmful and welcome the reassessment of its use as a therapeutic strategy for preserving insulin sensitivity. Despite observing no changes in mitochondrial content, function, or maximal H$_2$O$_2$ emission in isolated mitochondria, LA maintained skeletal muscle insulin signaling similarly to ALA. Future investigations should focus on changes in membrane phospholipid composition, as both n-3 and n-6 PUFA are known to compete in the remodeling of membranes, including mitochondria, and may also preferentially accumulate in different tissues. The impacts of LA and ALA on IR may transcend the boundaries of skeletal muscle and mitochondria but nevertheless represent valuable therapeutic strategies for preventing the development of an insulin-resistant phenotype in obesity.

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